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# Specific PCR Amplification for the Genus *Pseudomonas* Targeting the 3' Half of 16S rDNA and the Whole 16S–23S rDNA Spacer

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## Summary

A PCR protocol was developed for the selective amplification of a segment of the ribosomal RNA operon in *Pseudomonas* strains. Two specific conserved sequences suitable for PCR priming were identified in the middle of the 16S rDNA and at the very beginning of the 23S rDNA respectively. As a result, amplified region includes the 3' half of the 16S rDNA with the whole 16S–23S rRNA Internal Transcripted Spacer (ITS1) sequence. The specificity of the primer set was checked on sequence databases and validated on collection strains and on one hundred soil bacterial isolates. Our results showed that both collection, soil-inhabiting *Pseudomonas* and some *Pseudomonas*-related *Azotobacter* DNAs could be amplified. This specific PCR for the detection of *Pseudomonas* strains was in good agreement with colony hybridisation using a *Pseudomonas*-specific probe. The targeted segment is relevant for a characterisation at the species (16S rDNA) as well as at the infraspecific (ITS1) levels. This PCR-based approach offers promising potential for the characterisation of environmental *Pseudomonas* populations.

**Key words:** *Pseudomonas* – detection – specific primers – 16S–23S rDNA Intergenic Transcribed Spacer – ITS1 – colony hybridisation – soil.

## Introduction

The genus *Pseudomonas* was originally described by Migula in 1894. This genus was redefined recently and its phylogenetic relationships with related groups or previously misnamed *Pseudomonas* spp. were elucidated using 16S rDNA sequences (KERSTERS et al., 1996). The genuine *Pseudomonas* currently comprise more than 50 species (ANZAI et al., 2000). Bacteria belonging to the genus *Pseudomonas* are widely dispersed in soil and water environments. The genus *Pseudomonas* also contains important pathogens for plants, fungi, animals and even human (LYCZAC et al., 2000), including opportunistic pathogens that have become more and more problematic in term of public health. Nevertheless, some strains showed promising properties for the promotion of plant growth, the inhibition of plant pathogens (WALSH et al., 2001) or the degradation of xenobiotic compounds (DAANE et al., 2001). Understanding the role and evolution of *Pseudomonas* populations requires a comprehension of their diversity. Reliable tools for the detection of the members of the genus *Pseudomonas* are therefore needed.

Historically, the identification of *Pseudomonas* strains relied only on morphological and physiological features. However, such phenotypic traits have proved to be variable at infraspecific level. Intraclonal phenotypic variation was also described in numerous *Pseudomonas* species (GREWAL and RAINEY, 1991; RAINEY and TRAVISANO, 1998). Furthermore, studies on environmental isolates revealed the limits of culture media selective for *Pseudomonas* (KRAGELUND et al., 1996; JOHNSEN and NIELSEN, 1999; AAGOT et al., 2001).

Molecular approaches based on oligonucleotide probing or selective PCR amplification were proposed for the detection and the identification of *Pseudomonas* spp. in both environmental and clinical samples. Group-specific probes targeted against *Pseudomonas*-specific region for universal genes (BRAUN-HOWLAND et al., 1993; LUDWIG

**Abbreviations:**
ITS1 – 16S–23S rDNA Intergenic Transcribed Spacer
RDP – Ribosomal Database Project

et al., 1994; AMANN et al., 1996) permitted differentiation of members of the genuine *Pseudomonas* from related genera. Recently, WIDMER et al. (1998) proposed a primer pair, which could be used for the amplification of a 16S rDNA gene fragment in *Pseudomonas* from environmental DNA extract. Similarly, DE VOS et al. (1997) developed primer sets targeting the functional *oprL* and *oprI* outer membrane genes for a specific detection of *P. aeruginosa* and fluorescent *Pseudomonas* spp. respectively.

Most of the oligonucleotides developed to date targeted the 16S rDNA sequence. The 16S–23S rDNA Internal Transcribed Spacer (named ITS1) sequence has evolved faster than the sequence of rDNA genes, because of its non-coding function. The ITS1 comprises conserved regions (generally corresponding to tRNA genes) as well as regions highly variable in length and sequence (GÜRTLER and STANISICH, 1996; GARCÍA-MARTÍNEZ et al., 1999). Therefore, one can expect this sequence to express differences between closely related organisms, especially at the infraspecific level. The corresponding DNA fragments can be amplified, taking advantage of conserved nucleotide regions in the flanking 16S and 23S rDNA sequences. Moreover, the target region may include a more or less large part of the 16S rDNA sequence, being suitable for a reliable taxonomic characterisation at higher level.

The objective of the present work was to develop a method allowing a rapid identification of isolates to the genus *Pseudomonas* during routine testing as well as their subsequent genotypic characterisation. For this purpose, a new set of PCR primers specific for *Pseudomonas* was developed, allowing the amplification of ITS1 together with a phylogenetically significant part of 16S rDNA. The validity of this PCR protocol was assessed on collection strains as well as environmental soil isolates.

## Material and Methods

All sequence positions presented in the current work are in accordance with positions corresponding to *Escherichia coli* gene (16S rDNA, 23S rDNA) numbering.

### Design of *Pseudomonas*-specific primers

Complete 16S rDNA sequences for 57 *Pseudomonas* species *sensus stricto* (ANZAI et al., 2000) and *E. coli* (accession number J01859), and 10 sequences available for the very beginning of the 23S rDNA gene for some *Pseudomonas* spp. and for *E. coli* (Figure 2) were retrieved from GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html). The 16S and 23S rDNA sequences respectively were aligned with GeneBase program (Applied Maths, Kortrijk, Belgium). This alignment was used to determine conserved regions for PCR primer design.

### Theoretical evaluation of *Pseudomonas*-specific primers

For each region, the most relevant oligonucleotidic sequence (named fPs16S for the forward primer on the 16S rDNA gene and rPs23S for the reverse one on the 23S rDNA gene) was selected for its conservation and specificity to *Pseudomonas* sequences.

Candidate sequences were 5′-ACTGACACTGAGGTGCGAAAGCG-3′ for fPs16S (position 756–779) and 5′-ACCGTATGCGCTTCTTCACTTGACC-3′ for rPs23S (position 1–25).

These two sequences revealed compatible melting temperatures (61.3 °C and 61.2 °C respectively, using the G/C rule, SAMBROOK and RUSSELL, 2001) and could be used as primers for PCR amplification using stringent conditions to ensure the specificity of the annealing.

The theoretical matchings of both sequences were assessed in the RDP database using CheckProbe analysis version 2.1r3 (MAIDAK et al., 2001), and in the GenBank database using the BLAST program (ALTSCHUL et al., 1997).

### Collection strains and environmental isolates

The collection strains used in this study and their source of isolation are listed in Table 1. They comprise 33 strains of numerous species and subspecies of *Pseudomonas* (Table 1a) as well as 17 strains of non-*Pseudomonas* (Table 1b).

Environmental isolates originated from a natural soil under a littoral meadow (at south shore of lake Neuchâtel, Switzerland). Total cultivable heterotrophic aerobic bacteria were recovered by tenfold serial dilutions of a soil suspension and plating on non-selective Angle medium (ANGLE et al., 1991). About one hundred Gram-negative isolates were randomly selected.

### DNA extraction

Overnight cultures of collection and environmental strains on Nutrient Agar were collected in sterile microtubes and washed by shaking for 4 min in 0.4 M NaOH. The bacterial cells were pelleted by centrifugation (13.000 g for 15 min), washed in TE buffer pH 8.0, and re-centrifuged in the same conditions. Bacterial cells were submitted to DNA extraction following the procedure described by MOORE et al. (1999), except that 15 µl of boiled 10 mg · ml$^{-1}$ RNAse was added during the proteinase digestion step.

### PCR amplification of ITS1

Two primer sets were used for PCR amplification of the ITS1 region (see Fig. 1): fPs16S/rPs23S (*Pseudomonas* primers defined in the present study) and S-D-Bact-1522-b-S-20/L-D-Bact-132-a-A-18 (*Bacteria* primers, NORMAND et al., 1996). For the *Pseudomonas*-specific PCR, the reaction mix contained (final concentrations): 1× Thermophilic DNA Buffer (Promega), 3 mM $MgCl_2$, 0.2 mM each dNTPs (Gibco), 0.25 µM each primer (Microsynth, Balgach, Switzerland), 0.05 U · µl$^{-1}$ Taq DNA polymerase (Promega), and 10% (vol:vol) of ten-fold diluted template DNA. The reactions were performed in a PTC-200 Peltier Thermal Cycler (MJ Research Inc., Massachussets) with an initial denaturation of 3 min at 94 °C, followed by 40 cycles of 1 min at 94 °C, 30 s at 55 °C and 1 min at 74 °C, and a final extension step at 74 °C for 5 min. PCR products were analysed by electrophoresis in 1.3 % agarose gel (Eurobio, Les Ullis, France) and visualised after staining with ethidium bromide. PCR amplification with *Bacteria* primers was used in order to check the quality of DNA extract and the size of ITS1 region. It was carried out as previously described (NORMAND et al., 1996).

### Hybridisation with $PSM_G$ probe

Bacterial colonies were tested for their hybridisation with $PSM_G$ probe (target position: 440–456 on 16S rDNA) specific for *Pseudomonas* spp. (BRAUN-HOWLAND et al., 1993) as previously described (MARILLEY et al., 1999).

### Cloning and sequencing of 16S rDNA fragments

16S rDNA genes were amplified with Gm3f and GM4r primers (MUYZER et al., 1995). PCR products were purified using Nucleotrap extraction kit for nucleic acids (Macherey-Nagel GmbH, Düren, Germany), and cloned into pGEM-T vec-

tor (Promega) and *E. coli* competent cells. Transformants were processed with NucleoSpin Plasmid kit (Macherey-Nagel) for plasmid extraction, as recommended by the manufacturer. The corresponding inserts were sequenced using T7 labelled primers (SAMBROOK and RUSSELL, 2001). The identification of the corresponding organisms was achieved by using a BLAST analysis on the retrieved sequences.

## Results

### Design of specific primers

The definition of *Pseudomonas*-specific primers was based on the sequences available in GenBank database. Target regions conserved and specific for the genus *Pseudomonas* (relatively to the available sequence data) were identified (Fig. 1) at locations 756–779 for 16S rDNA sequences (fPs16S), and 1–25 for 23S rDNA sequences (rPs23S). fPs16S and rPs23S sequences were conserved in the fifty seven 16S rDNA and the ten 23S rDNA *Pseudomonas* available sequences respectively. They displayed 2 and 5 mismatches with the 16S and 23S rDNA *E. coli* corresponding sequences, respectively.

### Theoretical evaluation of Pseudomonas primers

The fPs16S sequence displayed a perfect match with 551 sequences among 7322 16S rDNA prokaryotic sequences available in RDP database. We considered these matching sequences for their phylogenetic affiliation. Briefly, 66% of these sequences corresponded to *Pseudomonas* or uncultured and unidentified *Pseudomonas*-related sequences. Four sequences (less than 1%) were related to *Azotobacter* spp. (*Pseudomonadaceae*). About 19% corresponded to other γ-proteobacteria 16S rDNA sequences (*Oceanospirillum* group including *Halomonas* and *Marinomonas* genus, *Moraxellaceae*, sulfur-oxidizing symbionts and unidentified γ-proteobacteria). Less than 10% were retrieved from other bacterial groups: 26 sequences from *Rickettsiaceae* (α-proteobacteria) and 22 sequences from δ-proteobacteria (*Desulforhabdus*, *Desulfovibrio*, *Lawsonia* spp.). Four other sequences matching with fPs16S (Y13327, AF112477, Z29622, D88521) corresponded to misnamed organisms and were actually closely related to *Pseudomonas* spp. (identity >98%).

Only two 23S rDNA sequences related to *Pseudomonas* (among 1400 sequences from eukaryotic,

**Fig. 1.** Primers used for PCR-amplification of ITS1.
1* primer fPs16S (*Pseudomonas*, forward) position 756–779;
2** primer FGPS1472–900 (*Bacteria*, forward) position 1472–1490;
3* primer rPs23S (*Pseudomonas*, reverse) position 1–25;
4** primer FGPL132'38 (*Bacteria*, reverse) position 118–135;
* present study; ** NORMAND et al., 1996.
Positions are given according to *E. coli* numbering on 16S and 23S rDNA respectively.

| | 1 | 11 | 21 | 31 | | 41 |
|---|---|---|---|---|---|---|
| rPs23S (coding sequence) | | **GGTCAAGTGA** | **AGAAGCGCAT** | **ACGGT** | | |
| *P. fluorescens* (AF134704) | .......... | .......... | .....GGATG | CCTTG | GCAGT | CAGAGGCGA |
| *P. mendocina* (L28159, L28160, L28161) | .......... | .......... | .......... | ..... | ..RR. | ..S...... |
| *P. aeruginosa* (AE004502, Y00432) | .......... | .......... | .......... | ..... | ..... | ......... |
| *P. aeruginosa* (L28148, L28149, L28150) | .......... | .......... | .......... | ..... | ..RR. | ..S...... |
| *P. stutzeri* (U65012, X87289) | .......... | .......... | .......... | ..... | ..... | ......... |
| *P. perfectomarina* (L03788) | .......... | .......... | .......... | ..... | ..... | ......... |
| *Azotobacter vinelandii* (AF079809) | .......... | .......... | .......... | ..... | ..... | ......... |
| *Stenotrophomonas maltophilia* (L28166) | .......C.. | .T.......C | .......... | ..... | ..RR. | ..S...... |
| *Halomonas pacifica* (AJ306882) | .......C.. | TT........ | .......... | ..... | ....C | ......... |
| *Moraxella catarrhalis* (A27628) | ........A. | T....T.... | .T........ | ..... | ..... | ......... |
| *Vibrio vulnificus* (X87293) | ...T...... | CT.....T.C | .......... | ..... | ..... | ......... |
| *Escherichia coli* (V00348) | ...T...C.. | CT.....T.. | .......... | ..... | ..... | ......... |
| *Acinetobacter calcoaceticus* (X87280) | A.......A. | TT...T.... | GT........ | ..... | ..... | ......... |
| *Buchnera aphidicola* (U09230) | ...T...CA. | .T.....T.C | .T....A... | ..... | ..... | ......... |
| *Ehrlichia chaffeensis* (AF000721) | .....GA... | ..GTATCA.A | T.CTGAATCC | ATAG. | .GTT. | G.AGC.AAC |
| *Coxiella burnetii* (X79704) | ...AG..GAG | C.TTCT.T.A | C.T...A.G. | TGA.C | .AGAG | GTTT.CT.G |

**Fig. 2.** Multiple nucleotide alignment of the available 5' end of 23S rDNA sequences of *Pseudomonas* and fPs16S-matching non-*Pseudomonas* species. Nucleotides are identified for mispairings only. Nucleotides identical to the rPs23S sequence are indicated by dots. The positions are given according to *E. coli* 23S rDNA numbering. R – A/G, S – G/C.

**Table 1a.** *Pseudomonas* strains used in this study.

| Taxon | Collection number | Original source |
|---|---|---|
| *P. aeruginosa* | ATCC 10145 | unknown |
| *P. agarici* | ATCC 25941$^T$ | *Agaricus bisporus* |
| *P. asplenii* | LMG 2173$^T$ | *Asplenium nidus* |
| *P. chlororaphis* | ATCC 17415 | soil |
| *P. chlororaphis* | DSM 6698$^T$ | clay in kerosene |
| *P. blatchfordae* | ATCC 9446$^T$ | *Phaseolus vulgaris* |
| *P. caricapapayae* | ATCC 33615$^T$ | *Carica papaya* |
| *P. chlororaphis* | ATCC 9446$^T$ | plate contaminant |
| *P. cichorii* | ATCC 10857$^T$ | *Cichorum endiva* |
| *P. corrugata* | ATCC 29736$^T$ | *Lycopersicon esculentum* |
| *P. fluorescens* | ATCC 27663 | soil |
| *P. fluorescens* | CFBP 2022 | *Allium sativum* |
| *P. fluorescens* bv IV | ATCC 12983 | soil |
| *P. fluorescens* bv IV | ATCC 17513 | water |
| *P. fluorescens* bv III | ATCC 17400 | hen egg |
| *P. fluorescens* bv II | ATCC 17482 | unknown |
| *P. fluorescens* bv I | ATCC 13525$^T$ | water reservoir |
| *P. fluorescens* bv I | ATCC 17397 | tap water |
| *P. fluorescens* bv VI | ATCC 17552 | water |
| *'Pseudomonas gingeri'* | LMG 5327 | *Agaricus bisporus* |
| *P. marginalis pv marginalis* | ATCC 17819 | pleural fluid |
| *P. putida* biotype C | ATCC 17386 | water |
| *P. putida* biotype A | ATCC 12633$^T$ | soil |
| *P. putida* biotype B | ATCC 17430 | unknown |
| *P. tolaasii* | ATCC 33618$^T$ | *Agaricus bisporus* |
| *P. stutzeri* | ATCC 17588$^T$ | unknown |
| *P. fluorescens* | CHA0 | tobacco (Switzerland) [1] |
| *Pseudomonas* sp. | TM1A3 and TM1A4 | tomato (Switzerland) [1] |
| *Pseudomonas* sp. | PGNL1 and PGNR1 | tomato (Ghana) [1] |
| *Pseudomonas* sp. | FL3 | rhizoplane soybean [2] |
| *Pseudomonas* sp. | FL9 | rhizosphere of pea [2] |

[1] KEEL et al., 1996 ; [2] RAO and JOHRI, 1999.
CFBP – Collection Française de Bactéries Phytopathogènes
type strains are indicated by a $^T$ after the collection number

**Table 1b.** Non-*Pseudomonas* γ-proteobacterial strains used for primer set validation.

| Taxon | Collection number | Family |
|---|---|---|
| *Azotobacter chroococcum* | DSM 374 | *Pseudomonadaceae* |
| *Azotobacter chroococcum* | DSM 2286 | *Pseudomonadaceae* |
| *Azotobacter sp.* | DSM 1721, DSM 1722, DSM 1723 | *Pseudomonadaceae* |
| *Azomonas agilis* | DSM 375$^T$ | *Pseudomonadaceae* |
| *Pseudoalteromonas gracilis*[1] | H40 | *Alteromonadaceae* |
| *Vibrio fischeri* | DSM 507 | *Vibrionaceae* |
| *Enterobacter cloacae* | NEU 1027 | *Enterobacteriaceae* |
| *Enterobacter aerogenes* | DSM 30053 | *Enterobacteriaceae* |
| *Escherichia coli* | NEU 1006* | *Enterobacteriaceae* |
| *Klebsiella oxytoca* | NEU 30* | *Enterobacteriaceae* |
| *Proteus vulgaris* | NEU 1049* | *Enterobacteriaceae* |
| *Providencia alcalifaciens* | NEU 84* | *Enterobacteriaceae* |
| *Salmonella panama* | NEU 1065* | *Enterobacteriaceae* |
| *Serratia marescens* | NEU 1024* | *Enterobacteriaceae* |

* NEU : bacterial collection of the University of Neuchâtel
type strains are indicated by a $^T$ after the collection number
[1] MOEBUS (1992)

**Table 2.** Amplification results with fPs16S–rPs23S primers and $PSM_G$ hybridization of soil bacterial isolates.

| | PCR + | PCR– | total |
|---|---|---|---|
| H+ | 35 | 2 | 37 |
| H– | 0 | 64 | 64 |
| total | 35 | 66 | 101 |

H+/H–: isolates diplaying a positive/negative (respectively) hybridization with $PSM_G$ probe.
PCR+/PCR–: isolates displaying a positive/negative (respectively) amplification with fPs16S and rPs23S primers.

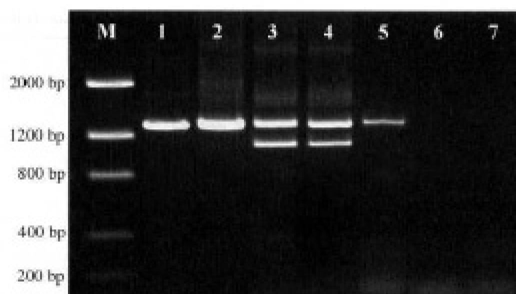


**Fig. 3.** PCR amplification of collection strain DNA with *Pseudomonas* primers. PCR was performed with fPs16S and rPs23S primers.
M – Low DNA Mass Ladder (Gibco), 1 – *P. asplenii* (LMG 2173), 2 – *P. fluorescens* (ATCC 17513), 3 – *P. fluorescens* (CFBP 2022), 4 – *P. marginalis* pv. *marginalis* ATCC 17819, 5 – *A. chroococcum* DSM 374, 6 – *E. coli*, 7 – control (no DNA template).

prokaryotic and mitochondrial origin) were available in the large ribosomal subunit RDP database. We assessed the target sequences of rPs23S using GenBank database. A BLAST analysis on rPs23S sequence displayed a perfect match for only 23 sequences in GenBank database. These included sequences from 16 identified *Pseudomonas* spp., one unidentified sugarcane isolate (AF251157) related to *Pseudomonas* (94% identity with *P. tolaasii*), two Chlamydia-associated clinical sample related to *P. aeruginosa* (99% of identity), three environmental clones (AF422501, AF4224999 and AF422492, related to *Pseudomonas* ITS1 sequences) and one *Azotobacter vinelandii* (AF079809). Figure 2 presents the alignment of available 23S rDNA sequences with particular interest for *Pseudomonas* and organisms for which 16S rDNA sequences displayed a perfect match with fPs16S.

When grouping sequences matching with both primers, all available non-*Pseudomonas* sequences in RDP and GenBank databases displayed at least 1 mismatch for at least one of the specific primers, except *Azotobacter vinelandii* (sequences L40329 for 16S rDNA and AF079809 for 23S rDNA).

### Experimental validation of fPs16S and rPs23S specific primers

*Pseudomonas* and non-*Pseudomonas* DNA extracts were tested for amplification using the fPs16S–rPs23S primer set. All the 33 *Pseudomonas* strains (Table 1a) were positively amplified using these primers and generated usually about 1300 bp PCR products. Some *Pseudomonas* strains generated 2 or 3 discrete bands after PCR amplification, whose size ranged from about 1100 to 1300 bp (Fig. 3). Multiple band patterns of these strains were confirmed by the use of ITS1 *Bacteria* primers. None of the 17 non-*Pseudomonas* collection strains (Table 1b) generated an amplicon with fPs16S and rPs23S primers, except *Azotobacter chroococcum* DSM374, for which a PCR product, at the expected size (about 1300 bp), was obtained. The four other *Azotobacter* strains were recorded as negative for PCR amplification with specific primers, although their DNA could be amplified with *Bacteria* primers.

One hundred and one Gram negative soil isolates were tested for amplification with fPs16S and rPs23S. The strains giving an amplification were recorded as positive (noted PCR+). When no amplicon was obtained, the strains were recorded as negative (noted PCR–). Thirty-five isolates were PCR+ and generated an amplicon at the expected size (1 to 3 fragments ranging from about 1100 to 1300 bp). Eight of these isolates were submitted to 16S rDNA sequencing and their affiliation to *Pseudomonas* genus was confirmed (identity > 94% using BLAST analysis). Three of the retrieved sequences were closely related to the 16S rDNA of *P. alcaligenes* (> 99% identity). One additional isolate, which generated unusual profiles after restriction analysis of the amplicon (data not shown) was also submitted to partial 16S rDNA sequencing and could be affiliated to *Stenotrophomonas maltophilia* (*Xanthomonas* group, γ-proteobacteria). Nonetheless, the 16S (i.e. AB008509) and 23S (L28166) rDNA sequences available in databases displayed several mismatches with fPs16S and rPs23S.

The reliability of fPs16S–rPs23S primers was assessed using hybridisation with the *Pseudomonas*-specific $PSM_G$ probe. The soil isolates were recorded as positive (H+) when hybridisation generated a strong signal and as negative (H–) when no signal or a faint signal was recorded. The comparison between specific amplification and $PSM_G$ hybridisation results is presented in Table 2. All PCR+ strains were also H+ and all the H– strains were PCR–. Two bacterial strains were recorded H+ but were negative for amplification with fPs16S–rPs23S. Partial 16S rDNA sequencing revealed that these strains were related to *Pantoea agglomerans* and *Pantoea ananatis* (100% identity on 755 bp and 99% identity on 735 bp respectively for BLAST analysis). When considering their 16S rDNA sequences, as well as *S. maltophilia*-related sequence, we could not retrieve the complete 15 bp putative hybridisation site for $PSM_G$ probe (only 9 to 10 bp length sequences displayed a match with $PSM_G$).

## Discussion

Limited data were available for the definition of a consensus oligonucleotide for *Pseudomonas* 23S rDNA. Such sequences were retrieved from sequences of the ITS1, which included the beginning of 23S rDNA (GILL et al., 1994; TYLER et al., 1995; GUASP et al., 2000), or from the few complete 23S rDNA gene sequences available for *Pseudomonas* (for instance LUDWIG et al., 1994). On the contrary, recent work on *Pseudomonas* taxonomy (MOORE et al., 1996; ANZAI et al., 2000) has given access, for the very first time, to a representative collection of 16S rDNA target sequences for this genus. A target region specific for *Pseudomonas* genus (fPs16S) was identified in the middle of the 16S rDNA sequence, allowing to include a phylogenetically meaningful part of the 16S rDNA gene in the amplified region. fPs16S and rPs23S, when combined, formed a primer set which was conserved for all available *Pseudomonas* sequences.

Theoretical matching with sequences in GenBank and RDP databases, as well as experimental testing on collection and environmental strains, have confirmed the specificity and efficiency of fPs16S and rPs23S for the specific amplification of 1100 to 1300 bp fragment(s) of the ribosomal operon in *Pseudomonas* spp. This primer set also revealed matches for *Azotobacter* spp. According to DE VOS et al. (1985), the genus *Azotobacter* is closely related to the genus *Pseudomonas*. Moreover, in the taxonomic hierarchy proposed by the RDP, *Azotobacter* spp. are included in the "*Pseudomonas* and relatives" cluster. Consequently, it was not surprising that some *Azotobacter* DNAs could be amplified with our primer set. As suggested by ANZAI et al. (2000), we recommend an extensive study for a definite conclusion on the taxonomy of this genus and its phylogenetic relationships with *Pseudomonas*.

In our study, some of the collection and environmental *Pseudomonas* strains yielded multiple size PCR products (up to 3), in agreement with previously published data (BENNASAR et al., 1998). For a given strain, the number of PCR products was identical with *Pseudomonas* and with *Bacteria* primers, confirming that the region targeted by our primer set included the ITS1. The ITS1 length varies both between species and between multiple operons in a given organism. Four rDNA transcriptional units have been described in *P. stutzeri* (GINARD et al., 1997) and *P. aeruginosa* (RÖMLING et al., 1989). Variations in the length of ITS1 are due, in part, to the number and type of tRNA genes that it may contain (for most of proteobacteria, including *Pseudomonas* spp.: $tRNA^{ALA}$ and $tRNA^{ILE}$) (JENSEN et al., 1993). The size of generated PCR fragments, which include about 750 bp of 16S rDNA sequence, is in good agreement with previously published data about the size of ITS1 for *Pseudomonas*: this may range from 515 to 548 bp (GILL et al., 1994; SAWADA et al., 1999; GUASP et al., 2000), when including both tRNA genes. In our study, limited length polymorphism was also detected for ITS1 sequences from a large collection of *Pseudomonas*.

Specific PCR amplification with fPs16S and rPs23S was in very good agreement with $PSM_G$ probing on soil isolates, as all the PCR+ strains were also H+. Nonetheless, a few H+ PCR– strains were not related to the genus *Pseudomonas*. These results suggested that the experimental (stringency) conditions were not optimal regarding the specificity of the probe.

The probing approach provides the information on the presence and numbers of bacteria affiliated to *Pseudomonas* genus (BRAUN-HOWLAND et al., 1993; MARILLEY et al., 1999). On the other hand, specific PCR amplification provides a reliable tool for (i) detecting *Pseudomonas* strains, (ii) confirming their affiliation to this genus and (iii) assessing their genotypic diversity, using the sequence variability of the generated PCR products.

The proposed approach could take advantage of the 16S rDNA fragment of the amplicon. This one displays a limited level of sequence variation or restriction polymorphism (BROSCH et al., 1996; ACHOUAK et al., 2000), and generates restriction fragments that are specific for some species or groups (LAGUERRE et al., 1994). Moreover, the ITS1 sequences were shown to be perfectly identical or very slightly different from strains belonging to a same biovar or genomovar, whereas they displayed frequent insertion or deletion events between strains from different subspecies (SAWADA et al., 1999; GUASP et al., 2000). The ITS1 region was also used for the definition of strain- or group-specific probes (GILL et al., 1994; TYLER et al., 1995) or PCR primers (YANG et al., 2000) in *Pseudomonas* genus, which can be useful for identification and monitoring purposes. Moreover, restriction analysis of ITS1 was shown to be valuable for the differentiation of *Pseudomonas* strains at an infra-specific level (MANCEAU and HORVAIS, 1997; CHO and TIEDJE, 2000; JENG et al., 2001).

Finally, preliminary results showed that the amplification of ITS1 fragments with fPs16S and rPs23S could be directly applied on environmental DNA extract. This feature seems particularly relevant for avoiding the bias of cultivability. Such an approach would be suitable for the monitoring of *Pseudomonas* populations in environmental samples, offering new promise in understanding the ecology of *Pseudomonas* organisms.

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